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Sustained intrahepatic glutathione depletion causes proteasomal degradation of multidrug resistance-associated protein 2 in rat liver

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ABSTRACT

Multidrug resistance-associated protein 2 (MRP2) is a member of a family of efflux transporters that are involved in biliary excretion of organic anions from hepatocytes. Disrupted canalicular localization and decreased protein expression of MRP2 have been observed in patients with chronic cholestatic disorder and hepatic failure without a change in its mRNA expression. We have previously demonstrated that post-transcriptional regulation of the rapid retrieval of rat MRP2 from the canalicular membrane to the intracelluar compartment occurs under conditions of acute (~30 min) oxidative stress. However, it is unclear whether MRP2 expression is decreased during its sustained internalization during chronic oxidative stress. The present study employed buthionine sulfoximine (BSO) to induce chronic oxidative stress in the livers of Sprague–Dawley rats and then examined the protein expression and localization of MRP2. Canalicular MRP2 localization was altered by BSO treatment for 2 h without changing the hepatic protein expression of MRP2. While the 8 h after exposure to BSO, hepatic MRP2 protein expression was decreased, and the canalicular localization of MRP2 was disrupted without changing the mRNA expression of MRP2. The BSO-induced reduction in MRP2 protein expression was suppressed by pretreatment with N-benzyloxycarbonyl (Cbz)-Leu-Leu-leucinal (MG-132), a proteasomal inhibitor. Furthermore, the modification of MRP2 by small ubiquitin-relatedmodifier 1 (SUMO-1) was impaired in BSO-treated rat liver,while that by ubiquitin (Ub) and MRP2 was enhanced. Taken together, the results of this study suggest the sustained periods of low GSH content coupled with altered modification of MRP2 by Ub/SUMO-1 were accompanied by proteasomal degradation of MRP2.

1. Introduction

Cholephilic compounds are excreted into the bile by distinct ATP-dependent primary active transporters located at the canalicular membrane. Of these, multidrug resistance-associated protein 2 (rat: rMRP2 and human: hMRP2) is of paramount importance. In humans, biliary excretion of organic anions (e.g., bilirubin glucuronides, reduced glutathione (GSH), and GSH conjugates) is mediated by MRP2, a conjugate export pump encoded by the MRP2 gene [1].

Several disorders are associated with deficient canalicular localization/expression of MRP2. For example, Dubin–Johnson syndrome (DJS) is a heredity disease characterized by conjugated hyperbilirubinemia. The deficient hepatobiliary transport of anionic conjugates results from the absence of a functional MRP2 in the canalicular membrane of hepatocytes. A number of mutations in the hMRP2 gene have been identified in patients with DJS. Certain DJS mutations (e.g., missence mutations R768W and I1173F; deletion mutation R1392, M1393) have been reported to cause defects in canalicular sorting and rapid proteasomal degradation of the hMRP2 protein [2–4]. In addition to the inherited abnormality of canalicular hMRP2 expression, it was reported that canalicular hMRP2 localization and overall cellular expression of hMRP2 protein was decreased without changing its mRNA level in patients with chronic liver failure (primary biliary cirrhosis and hepatitis C virus infection) [5,6] Notably, the presence of oxidative stress markers was correlated with chronic cholestatic disorder in these liver disease patients [7].

We have previously demonstrated that rMRP2 internalization from the canalicular membrane to the intracellular compartment occurs within a few minutes after ethacrynic acid (EA) treatment, tert-butyl hydroperoxide (t-BHP) treatment, or within 2 h after lipopolysaccharide (LPS) treatment [8–11]. Similar phenomenon was also confirmed for hMRP2 using liver slices [12]. All these events seem to be triggered by a

Abbreviations: ARC, apoptosis repressor with caspase recruitment domain; BSA, bovine serum albumin; Bsep/BSEP, bile salt export pump; BSO, buthionine-sulfoximine; CD26, cluster of differentiation 26; CrM, crude membrane; DJS, Dubin-Johnson syndrome; EA, ethacrynic acid; GADPH, glyceraldehyde-3-phosphate dehydrogenase; γ-GCS, γ-glutamyl cysteine synthetase; GSH, glutathione; HPLC, high performance liquid chromatography; LPS, lipopolysaccharide; MRP2, multidrug resistance-associated protein 2; PFIC, progressive familial intrahepatic cholestasis; PDZK1, PSD95/Dlg/ZO1-containing 1; PKC, protein kinase C; SUMO, small ubiquitin-like modifier; t-BHP, tert-butyl hydroperoxide; TTBS, Tween 20/Tris-buffered saline; Ub, ubiquitin; UBA2, ubiquitin-activating enzyme 2; Ubc9, ubiquitin-conjugating enzyme 9

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decrease of GSH in the hepatocytes. Based on our line of previous experiments using isolated rat hepatocyte culture model, we proposed that GSH decrease causes intracellular Ca^{2+} elevation, NO release and finally leads to PKC activation [9]. Then, interaction of rMRP2 with radixin, a cytoskeltal linker protein connecting rMRP2 to the filamentous actin, was decreased as a result of dephosphorylation of radixin [22]. We also demonstrated that once decreased rMRP2 surface expression induced by t-BHP was recovered to the control level by the replenishment of intracellular GSH within an hour [10]. On the other hand, only limited information is available on the fate of rMRP2 under sustained GSH decrease condition; i.e. stably pooled in the intracellular compartment to be recycled back to the cell surface or directed to degradation pathway.

Ubiquitination is a regulated post-translational modification that conjugates ubiquitin (Ub) to the lysine residues of target proteins, thereby determining their intracellular fate. The canonical role of ubiquitination is to mediate the proteasomal degradation of proteins that carry a single Ub molecule or a polymeric chain of Ub molecules on a specific lysine residue. Several proteins (transglutaminase 2 and apoptosis repressor with caspase recruitment domain (ARC)) are known to be degraded by ubiquitination during oxidative stress [14]. Recently, experiments employing the yeast-two hybrid screening demonstrated that small ubiquitin-like modifier (SUMO)-related enzymes and molecules, including ubiquitin-activating enzyme 2 (UBA2) and ubiquitin-conjugating enzyme 9 (Ubc9), interacted with the linker regions of rat and human MRP2 [15]. Moreover, modification of rMRP2 by SUMO-1 was confirmed (an isoform of the SUMO family member), and rMRP2 protein expression was decreased in Ubc9 knock-down rat hepatoma cells [15]. Although the fundamental role of SUMO has not yet been completely elucidated, it has been established that SUMO competes with Ub for modification of the same lysine residue in target proteins. In this manner, SUMO protects proteins from the Ub/proteasome-dependent degradation pathway [16–18].

The current study was designed to evaluate the hypothesis that SUMO-1/Ub-mediated modifications of rMRP2 are key factors that regulate the fate of rMRP2 during sustained GSH decreased condition. To examine this possibility, Sprague–Dawley rats were treated with buthionine-sulfoximine (BSO), an inhibitor of γ-glutamyl cysteine synthetase (γ-GCS). γ-GCS is a rate-limiting enzyme in the synthesis of GSH and as such, its inhibition can cause long-lasting GSH depletion for at least 8 h, which is longer than our previous experimental condition. The results of this study indicate that rMRP2 protein is internalized at early phase as reported previously and subsequently degraded at later phase in a proteasome-dependent manner if GSH decease is sustained for a while (8 h). Ubiquitination and SUMOylation of rMRP2 may have a role in determining its fate after internalization.

2. Materials and methods

2.1. Chemicals and reagents

BSO, *o*-phthalaldehyde, 2-mercaptoethanol, and trifluoroacetic acid were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). N-benzyloxycarbonyl (Cbz)-Leu-Leu-leucinal (MG-132) was obtained from Calbiochem (Darmstadt, Germany), and 3-fluorotyrosine was obtained from Tokyo Kasei Chemical Industry Co., Ltd. (Tokyo, Japan). Rabbit anti-MRP2 antiserum was raised against the 12-amino acid sequence at the carboxyl terminus of rat rMRP2 [19]. Mouse monoclonal antibody against MRP2 (M_2III6) was obtained from Chemicon International, Inc. (Temecula, CA). Monoclonal mouse antibody (FK2) against mono- and poly-ubiquitinated conjugates was obtained from Enzo Life Sciences, Inc. (Plymouth, PA). Purified mouse anti-rat cluster of differentiation 26 (CD26; OX-61) and mouse monoclonal anti-β-actin antibodies were obtained from Sigma-Aldrich Chemicals (St. Louis, MO). Rabbit polyclonal anti-SUMO-1 and SUMO-2/3 antibodies and horseradish peroxidase-conjugated secondary antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). All other chemicals and solvents were of analytical grade.

2.2. Animals

Male Sprague–Dawley rats (Japan SLC Inc., Shizuoka, Japan), 6 to 7 weeks of age and weighing 170 to 220 g, were used for all experiments. The animals were treated humanely in accordance with the "Guide for the Care and Use of Laboratory Animals" issued by the National Institutes of Health (Bethesda, MD). In addition, all protocols were approved by the Institutional Animal Care and Use Committee of Chiba University.

2.3. Experimental model

The rats were divided into two groups that each consisted of four to five rats. Experimental animals were given an intraperitoneal (i.p.) injection of BSO (1 mg/kg). Control animals were injected with saline instead of BSO. The rats were sacrificed 2, 8, or 24 h after BSO or saline injection, and the livers were harvested. In some experiments, the proteasome inhibitor MG-132 (0.5 mg/kg i.p.) was injected 1 h before BSO or saline treatment.

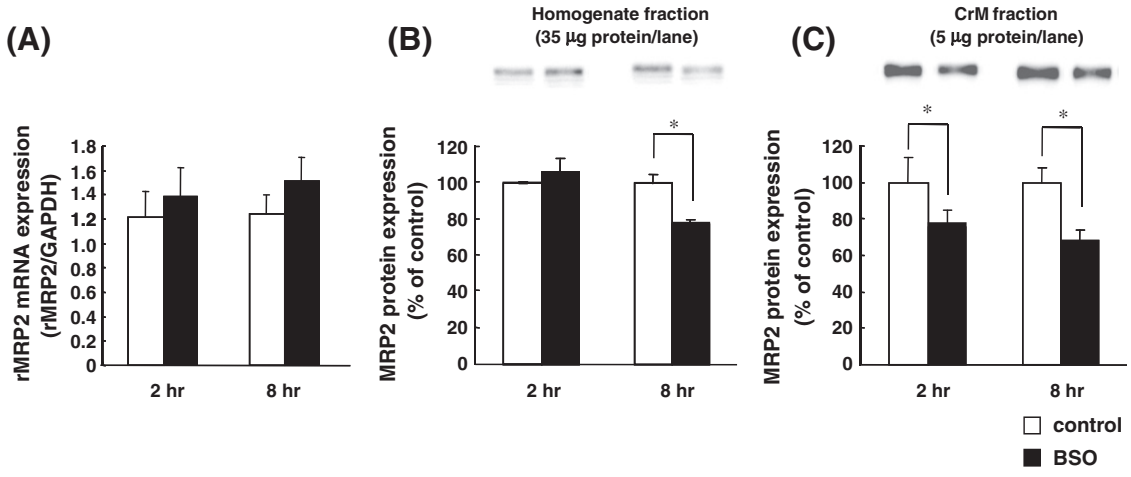

Fig. 1. Effect of BSO treatment on hepatic rMRP2 protein and mRNA expression. Rats were given BSO (1 mg/kg body weight) or saline by i.p. injection. (A) rMRP2 mRNA expression levels were measured by real-time RT-RCR. rMRP2 mRNA levels were normalized to GAPDH mRNA levels. (B, C) Crude liver homogenates (B) and CrM fractions (C) were subjected to immunoblot analysis with anti-MRP2 antiserum. The relative densities of the rMRP2 bands are shown. The band densities are expressed as a percentage of the corresponding saline-treated control value. Results are given as the mean ± S.D. (n = 4 independent rat livers for each condition.) *$p<0.05$ compared with control.

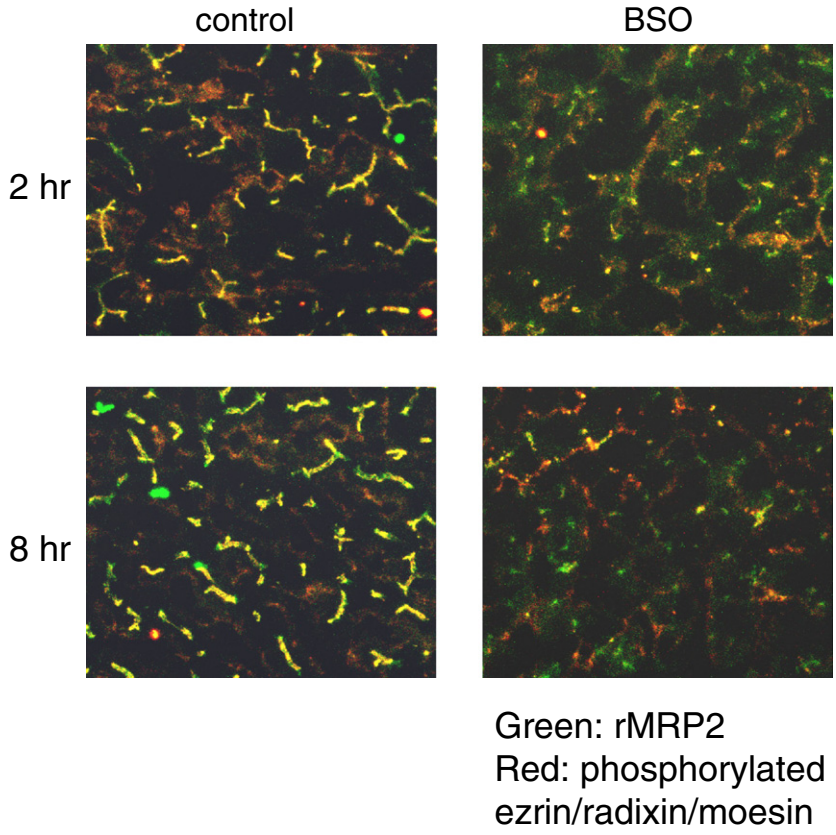

Fig. 2. Effect of BSO treatment on the distribution of rMRP2 and radixin in the rat liver. Immunofluorescence analysis confocal laser scanning microscopy of the rat liver. Frozen sections (6 μm in thickness) of acetone-fixed tissue were stained with anti-MRP2 (green) and anti-phosphorylated ezrin (Thr567)/radixin (Thr564)/moesin (Thr558) (p-ERM) antibody (red) antibodies and examined with a confocal laser microscope.

2.4. Measurement of hepatic intracellular GSH content

Liver samples were mixed with 3-fluorotyrosine as an internal standard followed by filtration through a 0.45-μm syringe filter (Millex-LH; Millipore Corp., Bedford, MA). High performance liquid chromatography (HPLC) was performed as described previously [20]. Briefly, an Inertsil ODS column (4.6-mm inner diameter × 250 mm; GL Sciences Ltd., Tokyo, Japan) was used with the mobile phase (0.1% trifluoroacetic acid to methanol ratio = 20:1) at a flow rate of 1.0 ml/min. The eluate from the column was mixed with a solution containing 1.86 mM *o*-phthalaldehyde and 1.71 mM 2-mercaptoethanol in 100 mM carbonate buffer (pH 10.5), which was delivered at a rate of 0.2 ml/min. The mixture was then passed through a stainless steel coil at 70 °C to facilitate derivatization of GSH with *o*-phthalaldehyde. A fluorescence detector was used and operated at an excitation wavelength of 355 nm and an emission wavelength of 425 nm. The concentration of GSH was calculated with reference to the height of a standard GSH sample.

2.5. Crude membrane preparation

Liver specimens (1 g) were snap-frozen in liquid nitrogen. The crude membrane (CrM) fraction was prepared as previously described [21]. CrM fractions and crude liver homogenates were subjected to immunoblot analysis, as described previously [8,9] and elaborated upon, below.

2.6. Immunoblot analysis

Rat liver CrM fractions (5 μg protein) and homogenates (35 μg protein) were subjected to electrophoresis in a 8.5% polyacrylamide slab gel containing 0.1% SDS and then transferred to an Immobilon-P Transfer Membrane filter (Millipore Corp., Billerica, MA). The membrane was blocked with TTBS (0.05% Tween 20/Tris-buffered saline) containing 3% bovine serum albumin (BSA) for 2 h at room temperature or overnight at 4 °C. Blotted proteins were probed with primary antibodies for 1 h at room temperature or overnight at 4 °C. TTBS containing 0.1% BSA was used to dilute the primary antibodies as follows: rabbit polyclonal antiserum against rat MRP2 [1:1000]; M_2III6 anti-MRP2 antibody [1:1000]; rabbit polyclonal anti-SUMO-1 antibody [1:1000]; rabbit polyclonal anti-SUMO-2/3 antibody [1:1000]; mouse monoclonal FK2 antibody against mono- and poly-ubiquitinylated conjugates [1:1000]; and mouse monoclonal anti-β-actin [1:2000]. The membrane was incubated for 1 h at room temperature with horse radish peroxidase-conjugated secondary antibodies that were diluted in TTBS containing 0.1% BSA. Immunoreactive proteins were detected using an enhanced chemiluminescence system (GE Healthcare, Little Chalfont, Buckinghamshire, UK) and an LAS-1000 luminescent image analyzer (Fujifilm, Tokyo, Japan).

2.7. Semiquantitative real-time RT-PCR

Total mRNA was prepared from perfused rat liver using the RNA Solve reagent (OMEGA Bio-tek, Inc., Doraville, GA). Reverse transcription was performed with total RNA (0.4 μg) using a Takara RNA PCR kit ver. 3.0 (Takarabio, Shiga, Japan). Real-time RT-PCR was performed using the Power SYBR Green PCR Master Mix (Eurogentee SA, Seraing, Belgium) to quantify the mRNA expression of rMRP2 (forward primer: 5′ tga tcg gtt tgc tga aga gct 3′; reverse primer: 5′ acg cac att ccc aac aca aa 3′) relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (forward primer: 5′ gtg gtg cca aaa ggg tca 3′; reverse primer: 5′ att tct cgt ggt tca cac cca 3′). Real-time RT-PCR amplification was determined utilizing an ABI Prism 7000 machine (Applied Biosystems, Foster City, CA).

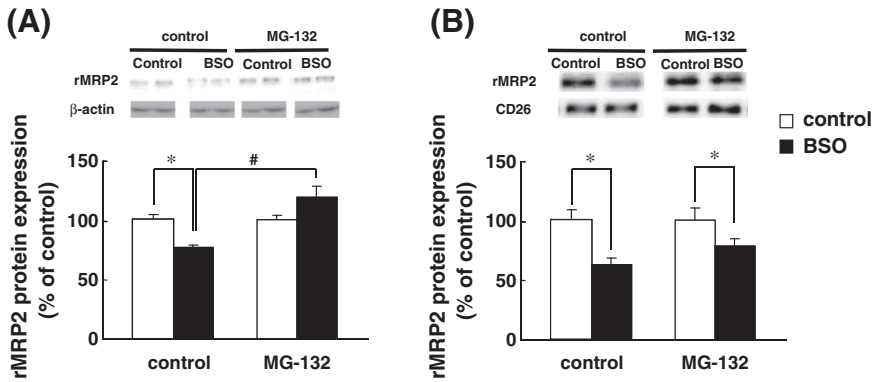

Fig. 3. Effect of the proteasomal inhibitor MG-132 on the BSO-induced reduction in rMRP2 protein expression. Rats were pretreated with MG-132 (0.5 mg/kg body weight) 1 h before BSO (1 mg/kg body weight) or saline treatment. (A, B) Crude liver homogenates (A) and isolated CrM fractions (B) were subjected to immunoblot analysis using an anti-MRP2 antibody. β-actin and CD26 were employed as loading controls. The relative densities of the rMRP2 bands are shown at 8 h after saline or BSO treatment. The rMRP2 band densities are expressed as a percentage of the corresponding saline-treated control value. Results are presented as the mean ± S.D. (n = 4 independently treated rat livers for each condition.) *$p<0.05$ compared with control. #$p<0.05$ compared with BSO.

2.8. Immunofluorescence analysis

Frozen small liver blocks were embedded in Tissue-Tek O.C.T Compound (Sakura Finetechnical Co. Ltd., Tokyo, Japan) and then used to prepare 6-μm-thick sections at −25 °C, which were then fixed in acetone at room temperature for 10 min. The sections on the slides were hydrated in 30 mM glycine containing phosphate-buffered saline (G-PBS) for 15 min. Then they were incubated with mouse monoclonal anti-MRP2 antibody M_2III_5 (Abcam, Cambridge, UK) (1:35) and rabbit anti-phosphorylated ezrin (Thr567)/radixin (Thr564)/moesin (Thr558) (p-ERM) antibody (Millipore Corp., Billerica, MA) (1:35) for 1 h. After rinsing with G-PBS, the slides were incubated for 1 h with goat Alexa Fluor 546 anti-rat IgG (1:150) and goat Alexa Fluor 488 anti-mouse IgG (1:150). The antibodies were diluted in PBS containing 0.1% BSA. After rinsing with G-PBS, the slides were dipped in TBS for 5 min and then the samples were mounted in VECTASHIELD (Vector Laboratories, Burlingame, CA). Immunofluorescence imaging was performed using a confocal laser scanning microscope, LSM510 type (Carl Zeiss, Jena, Germany).

2.9. Immunoprecipitation analysis

Frozen livers were homogenized with ten vertical strokes of a Teflon homogenizer (DuPont, Wilmington, DE) in lysis buffer (1 ml/g tissue wet weight) containing 25 mM Tris–HCl, pH 7.5, 5 mM EDTA, 250 mM NaCl, 1% (v/v) Triton X-100, 60 mM n-octyl-β-D-glucopyranoside, 50 mM NaF, 1 mM Na_3VO_4, and protease inhibitor solution (1 mM PMSF, 5 μg/ml leupeptin, and 1 μg/ml pepstatin A). Liver homogenates were incubated with lysis buffer (25 μl/mg protein) for 1 h at 4 °C and centrifuged at 20,630 g for 10 min. The supernatant was collected and rotated overnight at 4 °C with protein G-conjugated magnetic beads and anti-MRP2 antiserum (12 μl per 20 mg tissue homogenate protein). The beads were collected by sedimentation at 9,170 g for 1 min and washed three times with wash buffer containing 25 mM Tris–HCl, pH 7.5, 5 mM EDTA, 150 mM NaCl, 1% (v/v) Triton X-100, 50 mM NaF, and 1 mM Na_3VO_4. Finally, elution buffer containing 10 mM Tris–HCl, pH 6.5, 3% (w/v) SDS, 10% (v/v) glycerol, 5% (v/v) β-mercaptoethanol, 8 M urea, and 0.001% bromophenol blue was added, and the beads were boiled at 95 °C for 5 min. The suspension was centrifuged, and the supernatant was subjected to SDS-polyacrylamide gel electrophoresis followed by immunoblot analysis with M_2III6 FK2, anti-SUMO-1, and anti-SUMO-2/3 antibodies.

2.10. Statistical analysis

All data are represented as the mean ± the standard deviation (S.D.). Data were analyzed using the *t*-test (Figs. 1, 4, 5) and the Bonferroni correction (Fig. 3). Differences between the means at the level of $p<0.05$ were considered to be significant.

3. Results

3.1. The effect of BSO treatment on intrahepatic GSH content

Rats were given an i.p. injection of BSO (1 mg/kg body weight) to selectively inhibit γ-GCS and establish a sustained decrease in hepatic intracellular GSH content. Intrahepatic GSH content was evaluated at 2, 8, and 24 h after BSO or control saline injection to see how long the GSH decrease continued. As shown in Table 1, GSH content in BSO- versus saline-treated rats was significantly decreased at 2 and 8 h. The GSH content was 54.2% of the corresponding control value at 2 h and 17.2% of the corresponding control value at 8 h, but returned to control levels at 24 h after BSO injection (Table 1). This result suggests that GSH decrease was significantly reduced at least for 8 h under this experimental condition. Notably, it is substantially longer than our previous experimental condition (~2 h) where total protein expression of rMRP2 was not changed [8–11]. So, we now tried to chase the fate of rMRP2 until 8 h after BSO treatment where GSH decrease was still continued.

3.2. Effect of BSO treatment on rMRP2 protein and mRNA expression

Previous work indicated that MRP2 localized at the canalicular membrane was rapidly retrieved to the intracellular compartment after EA, t-BHP, or LPS treatment, as reflected by the decrease in GSH levels without changes in total rMRP2 protein or mRNA expression [8–11]. Therefore, the current study similarly evaluated rMRP2 protein and mRNA expression following BSO injection. At 2 h after treatment, neither the rMRP2 protein content nor the rMRP2 mRNA level was decreased in the livers of BSO-injected rats compared with the saline-injected controls. Hepatic rMRP2 mRNA and protein levels in BSO-injected rats were 114.5% and 106.2% of the values in saline-injected rats, respectively (Fig. 1A and B). At 8 h after BSO treatment, protein expression of rMRP2 in the liver was significantly decreased, but mRNA expression was unaltered. The protein and mRNA levels at 8 h were 77.2 ± 2.1% and 121.2 ± 15.2% of saline-treated control values, respectively (Fig. 1A and B). These data indicate that the rMRP2 protein was degraded in a post-transcriptional manner, and that the degradation was stimulated by a continuous GSH decrease during an 8 h time period.

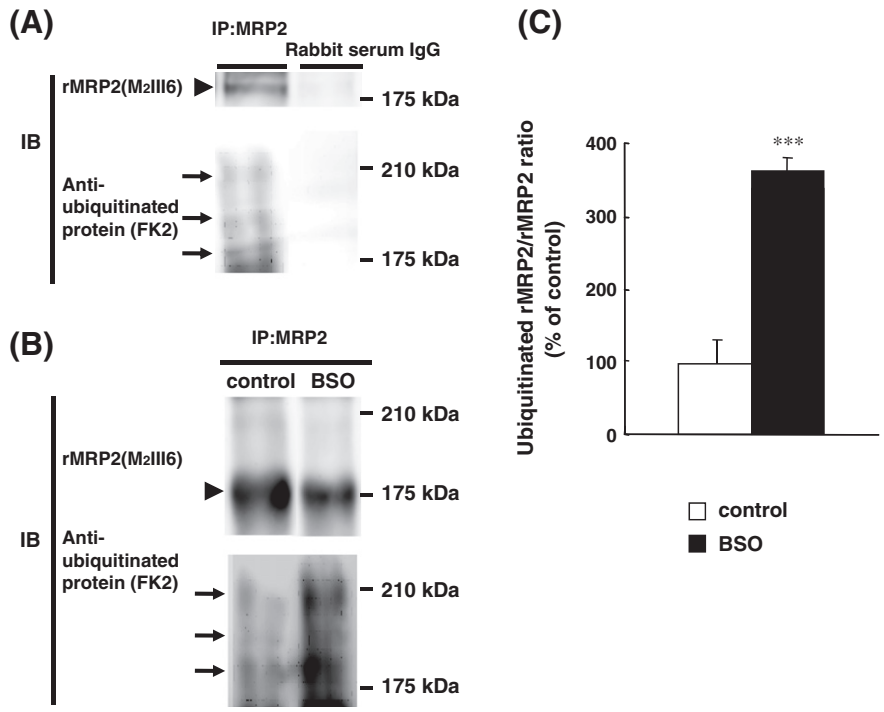

Fig. 4. Effect of BSO treatment on the modification of rMRP2 by ubiquitination. (A) and (B) Rat liver lysates were subjected to immunoprecipitation (IP) using anti-MRP2 antiserum. Immunoprecipitates derived from the livers of saline-treated (A) and BSO-treated (B) rats were then subjected to immunoblot (IB) analysis using monoclonal antibodies against MRP2 (M_2III6) and mono- and poly-ubiquitinylated conjugates (FK2). Rabbit serum IgG was used as a negative control in (A). (C) The band density of the ubiquitinated proteins in (B) (represented by arrows) was normalized by the band density of rMRP2. The values are expressed as the percentage of saline-treated control. Results are presented as the mean ± S.D. (n = 5 independently treated rat livers for each condition.) ***$p<0.001$ compared with control.

3.3. Effect of BSO treatment on the canalicular localization of rMRP2

As previously reported and noted above, canalicular rMRP2 localization is strictly correlated with intracellular GSH content [10,22]. Therefore, canalicular rMRP2 localization was investigated in BSO-treated rat livers. At 2 and 8 h after BSO injection, a decrease in rMRP2 expression was observed in the isolated CrM fraction. The rMRP2 expression at 2 h was 78.9 ± 2.8% of the control value in saline-treated rats, and further reduced to 65.7 ± 2.4% of the control value at 8 h (Fig. 1C). Hence, canalicular rMRP2 was internalized as early as 2 h following BSO treatment with no accompanying change in total hepatic rMRP2 expression (Fig. 1A and B). The decreased canalicular rMRP2 expression at 8 h after BSO treatment was apparently due to both the decrease in total rMRP2 protein expression (Fig. 1B) and the altered canalicular localization (Fig. 1C).

Immunofluorescence analysis of the liver treated with BSO supported these observation (Fig. 2). In control, rMRP2 colocalizes with phosphorylated radixin along the canalicular membrane as reported previously (reference 22). At 2 h after BSO treatment, colocalization was decreased and diffuse rMRP2 staining was observed. At 8 h after BSO treatment, overall rMRP2 fluorescence intensity was weakened compared to that in the sample at 2 h after BSO treatment.

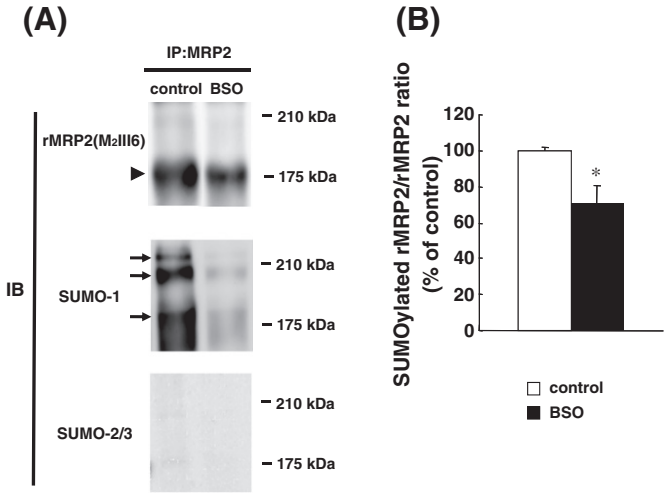

Fig. 5. Effect of BSO treatment on the modification of rMRP2 by SUMOylation. Rat liver lysates were subjected to immunoprecipitation (IP) using anti-MRP2 antiserum. (A) Immunoprecipitates were subjected to immunoblot (IB) analysis using anti-MRP2 (top; the same blot shown in Fig. 4B), SUMO-1 (middle), and SUMO-2/3 (bottom) antibodies. (B) The band density of SUMO-1 in immunoprecipitant (represented by arrows) was normalized by the band density of rMRP2 ((A) top). The values are expressed as a percentage of the saline-treated control. Results are presented as the mean ± S.D. (n = 5 independently treated rat livers for each condition.) *$p<0.05$ compared with control.

3.4. Role of the proteasomal degradation pathway

Most proteins undergo degradation through lysosomal and/or proteasomal complexes. The proteasome inhibitor MG-132 is cell-permeable and blocks proteasomal function, without affecting normal biological processes such as ATP metabolism and protein synthesis [23]. Because rMRP2 degradation was observed by virtue of the decline in intrahepatic GSH content at 2 and 8 h following BSO treatment, the dependence of rMRP2 protein degradation on the proteasomal pathway was next investigated using an in vivo experimental animal model.

Table 1
Effect of BSO on intrahepatic GSH content (nmol/mg protein).

	2 h	8 h	24 h
Control	28.4 ± 2.3	26.2 ± 2.5	27.2 ± 1.4
BSO	15.4 ± 1.5**	4.5 ± 1.0***	22.5 ± 3.8

The results are given as the mean ± S.D. of five rats. **$p<0.01$, ***$p<0.001$ compared with the saline control.

The dosing regimen of MG-132 used in this study (0.5 mg/kg i.p. injected 1 h before BSO or saline treatment) was based on a previous report, in which MG-132 selectively inhibited hepatic chymotrypticlike activity [24]. Pretreatment with MG-132 prior to saline treatment did not affect the protein expression of rMRP2 during the experimental period (8 h) as assessed by immunoblot analysis. The rMRP2 protein level in the pretreated liver was 102 ± 2.4% of the value in saline-treated control rats (Fig. 3A). However, the BSO-induced reduction in rMRP2 protein expression was significantly suppressed by pretreatment with MG-132, yielding an rMRP2 protein level that was 120 ± 9.3% of the value in the saline-treated control (Fig. 3A). On the other hand, β-actin expression in the total homogenate was not affected by the experimental conditions (Fig. 3A), demonstrating that the alterations in rMRP2 protein expression were not due to changes in total hepatic protein.

3.5. Effect of MG-132 on canalicular membrane localization of rMRP2 and intrahepatic GSH content

As discussed previously [10,22], hepatic GSH content is a major determinant for rMRP2 localization. Therefore, the effect of MG-132 pretreatment on intrahepatic GSH content and rMRP2 protein expression in the isolated CrM fraction was investigated. MG-132 did not affect GSH content in the livers of saline-treated control rats at 8 h after saline injection (Table 2). MG-132 also did not affect the extent of the GSH decrease in BSO-treated rats at 8 h after BSO injection (Table 2). A significant reduction in rMRP2 localized at the canalicular membrane was induced by BSO, irrespective of MG-132 pretreatment (Fig. 3B). By contrast, the BSO-induced reduction in rMRP2 protein expression in the liver homogenate was inhibited by MG-132 (Fig. 3A). These data suggest that MG-132 selectively inhibits the later degradation process of rMRP2 in the proteasome, but not the initial internalization process induced by GSH decrease per se.

3.6. Effect of BSO treatment on the modification of rMRP2 with Ub

Ubiquitination is a well-described post-translational modification. Ubiquitinated proteins are destined for degradation, especially in proteasomal complexes. This study next investigated the effect of BSO on the post-translational modification of rMRP2 with Ub. Eight hours after BSO or saline injection, liver lysates were subjected to IP with anti-rMRP2 antiserum, and immunoprecipitates were subjected to immunoblotting with monoclonal antibodies against MRP2 (M_2III6) or mono- and polyubiquitinated conjugates (FK2). The M_2III6-immunoreactive unmodified rMRP2 protein was detected as a 175-kDa band in saline-treated (control) and BSO-treated livers (arrowheads in Fig. 4A and B). The higher molecular weight bands indicated by arrows were observed with the FK2 antibody (Fig. 4A). These bands were also faintly visible with the M_2III6 antibody (Fig. 4B). Rabbit serum IgG was used as a negative control (Fig. 4A) and revealed no immunoprecipitated protein bands.

The overall density of the FK2-immunoreactive bands was significantly increased by BSO treatment compared with control saline treatment; the density was 191.1 ± 8.4% of the control value (Fig. 4B). On the other hand, the overall level of the rMRP2 protein was decreased by BSO treatment and was only 52.3 ± 15.2% of the control value (Fig. 4B), reflecting a decrease in its total expression at 8 h after BSO injection (Fig. 1B). Furthermore, the ubiquitinated rMRP2 to total rMRP2 ratio in the immunoprecipitated specimen was significantly increased by BSO treatment and was 365.5 ± 15.3% of the control value in saline-treated rats (Fig. 4C).

Table 2
Effect of MG-132 pretreatment and BSO on intrahepatic GSH content (nmol/mg protein).

	8 h
Control	27.4 ± 3.2
BSO	12.4 ± 2.6**
MG-132/saline	22.1 ± 5.4
MG-132/BSO	10.4 ± 5.5***

The results are given as the mean ± S.D. of five rats. **$p<0.01$, ***$p<0.001$ compared with the saline control.

3.7. Effect of BSO treatment on the modification of rMRP2 with SUMO-1

We have previously reported that SUMO-related proteins (Ubc9, UBA2, and SUMO-1) interacted with the linker region of rat and human MRP2 (rat: aa 851–958; human: aa 855–962) in yeast two-hybrid system [15]. In addition, decrease of rMRP2 protein occurred in Ubc9 knockdown rat hepatoma cells without affecting its mRNA expression [15]. These results proposed the possibility that MRP2 expression is regulated by SUMOylation. Therefore, the effect of BSO was next investigated on the modification of rMRP2 with SUMO-1 and SUMO-2/3 isoforms. rMRP2 antiserum-antigen immunoprecipitates from rat liver lysate were subjected to immunoblotting with a monoclonal antibody against MRP2 (M_2III6) or a polyclonal antibody against either SUMO-1 or SUMO-2/3. The rMRP2 content in the MRP2 antiserum-antigen immunoprecipitate (indicated by arrowhead around 175 kDa in Fig. 5A, top) was decreased at 8 h following BSO injection to 52.3 ± 15.2% of the saline-treated control value, which is consistent with the data shown in Figs. 3A. High molecular weight bands (indicated by arrows in Fig. 5A, middle) were observed with the SUMO-1 antibody. These bands were similarly decreased by BSO treatment to 38.3 ± 12.2% of the control value. However, no immunoreactive bands were detected with the SUMO-2/3 antibody (Fig. 5A, bottom). Furthermore, the ratio of SUMOylated to total rMRP2 ratio in the immunoprecipitated specimen was significantly decreased by BSO treatment to 73.2 ± 11.1% of the control value (Fig. 5B).

4. Discussion

The expression of efflux transporters including rMRP2/hMRP2 is regulated on a post-transcriptional as well as a transcriptional basis, with important implications for the pathogenesis of cholestatic syndromes. Post-transcriptional regulation of rMRP2/hMRP2 includes its rapid translocation from the canalicular membrane of hepatocytes into the intracellular space by LPS treatment [11,12,25], hyperosmolarity [26,27], phalloidin [28], bile acid [29], and oxidative stress [10,30,13]. However, the fate of rMRP2 after internalization and its regulatory mechanism, if any, have not yet been elucidated.

The current study demonstrated that BSO treatment induced time-dependent changes in rMRP2 localization/internalization and protein degradation in the rat liver. The expression of rMRP2 on the canalicular membrane was decreased 2 h after BSO treatment without changing rMRP2 total expression in the homogenate (Fig. 1B), as reported previously for EA, t-BHP, and LPS treatment [8–11]. At that short time point, rMRP2 is kept intact in the intracellular compartment, and possibly recycled back to the membrane surface if GSH is replenished as reported previously [10]. However, later time point at 8 h after BSO treatment, rMRP2 total protein expression in the homogenate was significantly reduced to 77.2% of the saline-treated control 8 h without changing rMRP2 mRNA expression. Considering that the half-life of rMRP2 protein in normal rat hepatocyte is reported to be approximately 29 h [31], rMRP2 protein degradation seems to be accelerated than usual under sustained GSH decreased condition (~8 h after BSO treatment). Collectively, these results and our previous observation [10] suggest that degradation of rMRP2 on the canalicular membrane surface follows two subsequent processes: 1) internalization process triggered by GSH decrease even by for short period [10], and 2) proteasomal degradation process promoted by ubiquitination under sustained GSH decrease condition (~8 h after BSO treatment; Fig. 6). Under such situation, it was observed that ubiquitination and SUMOylation of

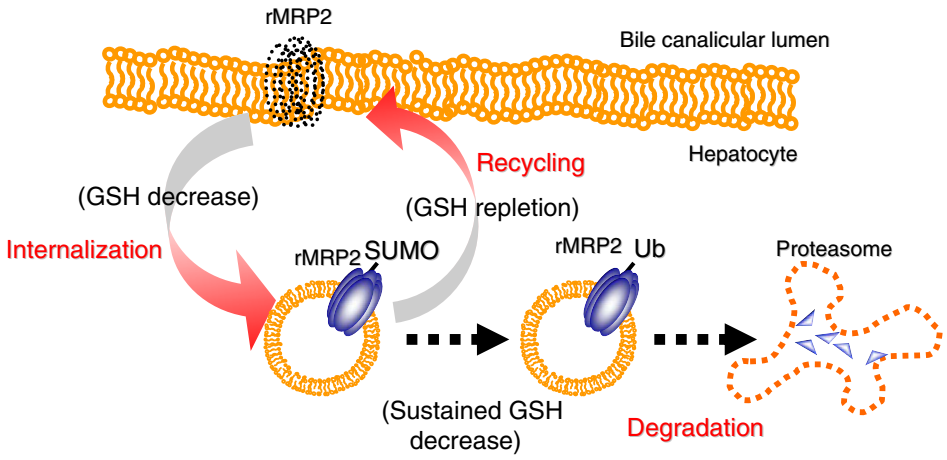

Fig. 6. Proposed scheme of rMRP2 degradation during the sustained decrease in GSH content. The results of this study indicate that the degradation pathway of rMRP2 during sustained decrease in GSH content is composed of two steps: 1) an early phase of internalization, and 2) a late phase of ubiquitin-dependent proteasomal degradation.

rMRP2 was increased and decreased, respectively. Considering the widely accepted concept that poly ubiquitination is a kind of "tag" for degradation [14] and our recent observation that SUMOylation of rMRP2 is likely involved in its increased protein expression [15], we propose the hypothesis that modification of rMRP2 by SUMO and Ub determine the fate of rMRP2 after internalizaion. It was reported that SUMO competes with Ub for the modification of target proteins on the same amino acid residue in some cases, thus protecting it from Ub-dependent degradation [32–34]. Such SUMO-mediated protection has been shown to occur for the glucose transporters GLUT1 and GLUT4 [35]. Although we did not determine the amino acid residue of rMRP2 modified by SUMO and Ub, such scheme is intriguing and consistent with our observation showing mirror image profile of rMRP2 SUMOylation and ubiquitination (Figs. 4 and 5).

Genetic alternations in canalicular transporters (MRP2 and BSEP) in human patients lead to conjugated hyperbilirubinemia and progressive liver diseases due to the intrahepatic accumulation of transporter substrates, respectively. For instance, abnormalities in the intracellular trafficking of MRP2 and BSEP facilitated their proteasomal or lysosomal degradations in patients with DJS and PFIC2, respectively [2–4,36,37]. A recent study indicated that BSEP can be ubiquitinated, and that this modification was associated with accelerated degradation rate of cell-surface-resident BSEP [38]. However, it remains unclear whether the accelerated degradation of cell-surface-resident BSEP as a result of ubiquitination stems from the promotion of endosomal sorting from the cell surface and/or delivery from the endosomal compartment to the lysosome. In the present study, it is also not yet known whether the modification of rMRP2 with SUMO-1 and Ubiqiutin occurs at the cell surface or in the endosomal compartment and its physiological meaning. Interestingly, it was also notable that SUMOylated rMRP2 was readily detected from cell homogenate but not from crude membrane under normal condition (i.e. without BSO treatment) (data not shown). This observation supports the hypothesis that conjugation reaction of rMRP2 with SUMO-1 or ubiquitin takes place in the endosomal compartment rather than on the cell surface. Considering the fact that rMRP2 also resides in the intracellular pool to some extent [29,13] and sorting from this pool to the cell surface occurs by several stimuli including hypo-osmolarity [26,27], blocking rMRP2 in the intracellular pool from degradation is one of the possible roles of SUMO modification. We have previously reported that rMRP2 internalized in response to acute oxidative stress can be restored to the canalicular membrane surface by the replenishment of intrahepatic GSH content [10]. Continuously accelerated internalization of rMRP2 due to sustained GSH decrease (~8 h after BSO treatment; Table 1) somehow switches the modification of rMRP2 from SUMOylation-dominant to ubiquitination-dominant, and finally Ub-dependent proteasomal degradation would be occurred (Fig. 6). These modifications may be important determinants for the fate of rMRP2 beneath the membrane surface (proteasomal degradation or recycling to the canalicular membrane surface).

5. Conclusions

In conclusion, this is the first demonstration of proteasomal degradation of rMRP2 during sustained conditions of reduced intracellular GSH (~8 h after BSO treatment). Modification of rMRP2 by ubiquitination and SUMOylation might be affected and relate to its proteasomal degradation. This information can be useful to understand the molecular mechanism of the regulation of rMRP2 expression under various pathological conditions.

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